Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Nathwani AC, Tuddenham EGD, Rangarajan S, et al. Adenovirus-associated virus vector–mediated gene transfer in hemophilia B. N Engl J Med 2011;365:2357-65. DOI: 10.1056/NEJMoa1108046.

Supplementary Appendix

Supplement to: Nathwani et al. Adeno-Associated viral vector mediated gene transfer for hemophilia B.

Contents

Supplementary Methods

In vivo transduction inhibition assay. 50µl of test plasma was administered into NOD-SCID mice via a tail vein injection. Mice in the positive control (AAV8 +ve plasma) cohort were injected with 50 µl of plasma from a macaque that had been transduced with AAV8 vector before and was known to have generated a neutralising anti-AAV8 antibody response. Negative control mice (AAV8 –ve plasma) received rhesus plasma from an animal that had been screened previously and found to be AAV8 antibody negative. An additional cohort of mice (PBS control) received 50 µl of PBS instead of plasma. Three hours after injection of plasma, $1x10^{10}$vgs of scAAV2/8-LP1-hFIXco were administered into the tail vein of each mouse. Plasma samples were obtained 5 days after administration of vector and assessed for expression of human FIX antigen. To normalise for variations between experiments, the absolute human FIX antigen levels in the experimental animals were divided by the human FIX values in the PBS control.

Anti-AAV8 specific ELISA. An immunocapture assay was used to detect anti-AAV8 specific antibodies in murine plasma as described before.[10] In brief, plates were coated overnight with an AAV 8 specific peptide (qttggtantqtlgfsqggpntmanqak) and/or AAV8 viral particles and then washed and blocked with PBST containing 6% BSA for 1 hour at 37^{o}C. After washing with PBST, 50 µl of dilutions of human plasma (1:50, 1:500, 1:1000) were then applied to these wells in duplicate. Antibodies against AAV8 were detected with a horseradish peroxidase conjugated anti-human IgG (Sigma, Poole, UK). Results were expressed as the end-point titre in relative units (RU/ml), defined as the reciprocal of the interpolated dilution with an absorbance value equal to five times the mean absorbance background value.

FIX:C measurement. Patient plasma samples were assayed using a standard one-stage APTT based assay on an ACL3000 (Instrumentation Laboratory, Bedford, USA). Briefly, patient samples were diluted 1:5, 1:10, 1:20 in Owrens buffered saline (OBS) and compared to a plasma laboratory standard calibrated against the 3rd international plasma standard (99/826) (NIBSC, Potters Bar, UK) that was diluted 1:5, 1:10, 1:20 and 1:40 in OBS. Patient dilutions were added to lyophilized hereditary FIX deficient plasma (Technoclone, Vienna, Austria) and APTT lyophilized silica reagent (Instrumentation Laboratory, Bedford, USA), incubated 5 minutes then clotted with $CaCl_2$. FIX:C results were reported as percentage of normal and IU/dl.

Vector titration. Formulated and vialed vector was titrated by the method of Fagone, et al.[15] Briefly, test article was thawed and mixed 1:1 with an agarose gel loading buffer which included sodium dodecyl sulphate and a quantitative reference DNA. After heating and cooling, samples were electrophoresed on native agarose gels, which were subsequently stained and imaged. The quantity of vector genomic DNA in the test article was evaluated by normalizing the digital signal of the vector genome band with the in-lane reference DNA standard, and comparing that signal with similarly normalized signals from mass standards loaded in separate lanes of the gel.

Cellular Immunology studies. T cell responses were measured using one-color ELISpot assay for IFN-γ as previously described.[8, 9, 23] For this assay, ELISpot plates pre-coated with an anti-human IFN-γ antibody were used (Mabtech). The day of the assay, plates were washed four times with 1x PBS and blocked with AIM-V (Invitrogen Gibco) supplemented with 3% heat inactivated (HI) FBS (Hyclone) for at least two hours at room temperature. After the blocking step plates were washed twice with AIM-V 3% HI-FBS medium and

antigens and mitogens were added to wells in 100 µL of volume. Each condition was tested in triplicate unless specified differently. Just prior to the assay, cryopreserved PBMC were thawed in a 37°C water bath and quickly transferred into culture medium (AIM-V 3% HI-FBS) containing 10U/mL Benzonase (EM Science), washed twice in order to eliminate residual DMSO and counted using a Countess automated counter (Invitrogen). Trypan blue exclusion was used to evaluate cell viability.

After counting, PBMC were adjusted to concentration of 1-2.5x10^6 cells/mL (depending on the cell recovery) in AIM-V 3% HI-FBS culture medium lacking Benzonase, and 100 µL of the cell suspension was carefully added dropwise to the wells containing antigens and mitogens. The assembled ELISpot plates were then wrapped in foil and placed in a 37°C 5% CO_2 incubator overnight or for at least 20 hours.

After incubation, excess medium was removed and 200µL of ice-cold water were added to the ELISpot plates. After 10 minutes incubation on ice, plates were washed 6 times with DPBS containing 0.05% Tween-20 followed by an additional wash with PBS.

An anti-human IFN-γ biotinylated antibody (Mabtech) was then added to the plate (100 µL per well of a 1:1000 dilution in PBS 1% BSA) and incubated for 2 hours at room temperature. Plates were then washed 6 times with PBS/Tween as described above and once with PBS only. 100 µL of streptavidin-alkaline phosphatase (Mabtech) diluted 1:1000 in DPBS 1% BSA was then added to each well. After 1 hour at room temperature, plates were washed four times with PBS/Tween and twice with PBS. Spots were detected by adding chromogenic substrate to the plates (BCIP/MBT, KPL). Color development was stopped by multiple washes with distilled water. Plates were then dried overnight at room temperature and stored at room temperature protected from light until analyzed. The spots in each well were enumerated using an ELISpot reader (Cellular Technologies Inc) and analyzed with specific software (Immunospot, Cellular Technologies Inc).

T cell reactivity was tested against the following antigens and controls:

A library of 15-mers overlapping by 10 amino acids in sequence was designed to span the entire AAV8 VP1 protein for a total of 146 peptides (Mimotope). Individual peptides were resuspended in 50% acetonitrile 0.1% acetic acid to a final stock concentration of 5mg/mL. Peptides were organized in 6 pools (AAV-P1-6) and tested at a working concentration of ~3µg/ml per peptide.

Similarly, a library of 15-mers overlapping by 10 amino acids in sequence was designed to span the entire human FIX protein for a total of 91 peptides (Mimotope). Individual peptides were resuspended in 50% acetonitrile 0.1% acetic acid to a final stock concentration of 5mg/mL. Peptides were organized in 3 pools (FIX-P1-3) and tested at a working concentration of ~3 µg/ml per peptide. Alternatively, recombinant FIX (Benefix, Wyeth/Pfizer) was used in the assay at a working concentration of ~20µg/ml. Lymphocyte culture medium was used as negative control.

Three positive controls were used in the IFN-gamma ELISpot assay, CEF (a pool of epitopes from CMV, EBV and flu viruses binding to several common HLA alleles from Mabtech), a mix of PMA (0.05 µg/mL) and ionomycin (1 µg/mL), and an anti-CD3 antibody (Mabtech). For the polyfunctional analysis of T cell responses using flow cytometry SEB (Staphylococcus enterotoxin B, Sigma) 1µl/ml (10mg/ml stock) was used.

DNA from the participants enrolled in the study was used for MHC class I and II HLA typing. HLA typing was performed at the University of Pennsylvania Medical Center, Department of Pathology and Laboratory Medicine or at the clinical trial site.

Entry criteria:

Inclusion was limited to subjects who are or have:

1. Males ≥ 18 years of age with established severe HB (FIX:C<1u/dl) resulting from a missense mutation in the hFIX gene which has not been associated with an inhibitor in the

database (http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html) with detectable FIX in serum. Individuals with a stop codon mutation, a promoter mutation, a small insertion or deletion causing a frame shift, a small in frame insertion or deletion or a splice junction mutation could be enrolled if their mutation had not been associated with an inhibitor,

2. Treated/exposed to FIX concentrates for at least 10 years,

3. A minimum of an average of 3 bleeding episodes per year requiring FIX infusions or prophylactic FIX infusions because of frequent prior bleeding episodes,

4. Able to give informed consent and comply with requirements of the trial,

5. Currently free of inhibitor and have no history of inhibitors to FIX protein, and

6. A negative family history for the development of an inhibitor,

7. Willing to practice a reliable barrier method of contraception.

Patients with any of the following were excluded: :

1. Evidence of active infection with Hepatitis B or C virus as reflected by HBsAg or HCV RNA positivity, respectively. To be considered negative for active infection, two negative assays at a minimum of a six month interval were required,

2. Exposure to Hepatitis B or C and on antiviral therapy,

3. Serological evidence of HIV or HTLV infection,

4. Significant liver dysfunction as defined by an abnormal ALT (alanine transaminase), bilirubin, alkaline phosphatase or INR. Potential participants who have had a liver biopsy in the past 3 years were excluded if they had significant fibrosis of 3 or 4 as rated on a scale of 0-4

5. Coronary artery disease as a co-morbid condition,

6. Platelet count of <150 x 109/l,

7. Creatinine $\geq$ 1.5 mg/dl,

8. Hypertension with systolic BP consistently ≥ 130mmHg or diastolic BP consistently ≥ 90mmHg,

9. History of active tuberculosis, fungal disease or other chronic infection,

10. History of chronic disease adversely affecting performance,

11. Detectable antibodies reactive with AAV8,

12. Subjects who were unwilling to provide the required semen samples,

13. Poor performance status (WHO performance status score >1) or

14. Received an AAV vector previously or any other gene transfer agent in the previous 6 months.

Statistical Analysis: The design of this study was similar to a Phase I dose finding study and, therefore, no sample size justification is provided. The participants were followed in a longitudinal manner and all the relevant information was collected. The analysis of the data was descriptive in nature but data collected in a longitudinal manner was analyzed using longitudinal methods such as mixed effect model which takes into account the correlation among the observation taken at various time points within a participant. Efficacy was a secondary endpoint, defined as persistence of biologically active FIX at ≥ 3% of normal levels. Expression of FIX was assessed frequently during the study period but only after a minimum of 72 hours had elapsed since the last infusion of FIX protein concentrates. Analysis of neutralising antibody response and other immunological parameters as well as viral shedding was primarily descriptive and involved both inter-participant and intra-participant comparisons across doses.

Supplemental Results

Vector shedding studies: The scAAV2/8-LP1-hFIXco vector genome was detectable in the plasma, saliva, semen and stools within 72 hours of vector infusion and up to but not after day 15 in all participants, with the exception of participant 1 whose semen remained clear of proviral DNA at all-time points assessed (Figure 1S). The amount of scAAV2/8-LP1-hFIXco vector DNA in the body fluids roughly correlated with the vector dose administered, as the highest level of viraemia ($2.5\text{-}6.2x10^{6}$vg/µl) was observed in the two participants that received $2x10^{12}$vg/kg on the first day post gene transfer. Vector sequences were not detected in the urine of any of the participants at any time point after administration of vector.

Humoral immune response to AAV8 capsid: The kinetics of the humoral immune response to AAV8 capsid after peripheral vein administration of scAAV2/8LP1-hFIXco was similar in all six participants and did not appear to be significantly influenced by the vector dose (Figure 2 and Table 1). Anti-AAV8 IgM antibodies peaked between 7 and 28 days after gene transfer and then declined to base line levels by 6 weeks. Anti-AAV8 IgG titers increased from baseline values (11±6 RU/ml) to reach peak values (3075±1452 RU/ml) at an average of 5 weeks and were then stably maintained for > 200 days. Anti-AAV8 IgG1 levels increased in parallel with total IgG values whilst IgG2 and IgG3 levels did not rise significantly above baseline (Figure 2). This pattern is consistent with a primary immune response to AAV8. Neutralizing antibody titer (NAB) as assessed by the mouse transduction inhibition assay, increased in parallel with the anti-AAV8 IgG levels in all participants (data not shown). The magnitude of humoral immunity between participants was different with participant 3 showing the highest increase in Anti-AAV8 IgG levels. All participants had anti-AAV2 IgG antibodies detected by ELISA (mean = 24±11 RU/ml) and transduction inhibition assay at the time of vector administration.

Supplemental Figure Legend

Supplemental Figure 1: Virus shedding post scAAV2/8-LP1-hFIXco administration in HB participants. A qPCR based assay was used to detect vector sequences in body fluids (plasma, [redline], stool. [brown line], urine [yellow line] saliva [gray line] and semen [blue line]) collected from each participant on the stipulated days after peripheral vein administration of vector. The PCR primers were specifically chosen to amplify a region within the codon-optimized FIX transgene. Standards consisted of serial dilutions of scAAV2-8-LP1-hFIXco in naïve human plasma. Negative samples were spiked with vector plasmid and subjected to PCR to ensure that the sample did not inhibit the PCR reaction.

Supplemental Figure 2: A summary of the serum ALT levels in participants in the low dose and intermediate dose cohorts

Supplemental Figure 3: Bioinformatics analysis of results in participants 5 and 6.

A. Histograms represent the results of the week 8 IFN-γ ELISPOT for Participant 5. PBMC were tested against six pools of peptides derived from the AAV8 capsid protein VP1 (AAV-P1-6), recombinant FIX Benefix, or alternate reading frame products of translation of FIX (ARF-1-3). Results are expressed as spot forming units (SFU) per million PBMC plated in the assay. Threshold for positivity is indicated by the horizontal line and is defined as 3X the medium control spot count and at least 50 SFU per million PBMC. Participant 5 showed high reactivity to AAV8 peptides at week 8, with maximum reactivity to pool 2. HLA typing for this participant was used in conjunction with bioinformatics tools as described before[8] to determined candidate epitopes, shown in red boxes in the peptide pool diagram on the right.

Two of the epitopes identified, which are predicted to bind to HLA-A*0201 alleles were contained in AAV8 pool 2, the pool which gave the highest reactivity in this participant. It should be noted that the participant showed poor reactivity to the CEF peptide pool, a positive control used in the assay. This was a consistent finding for this participant, indicating poor reactivity to the control rather than poor cell viability.

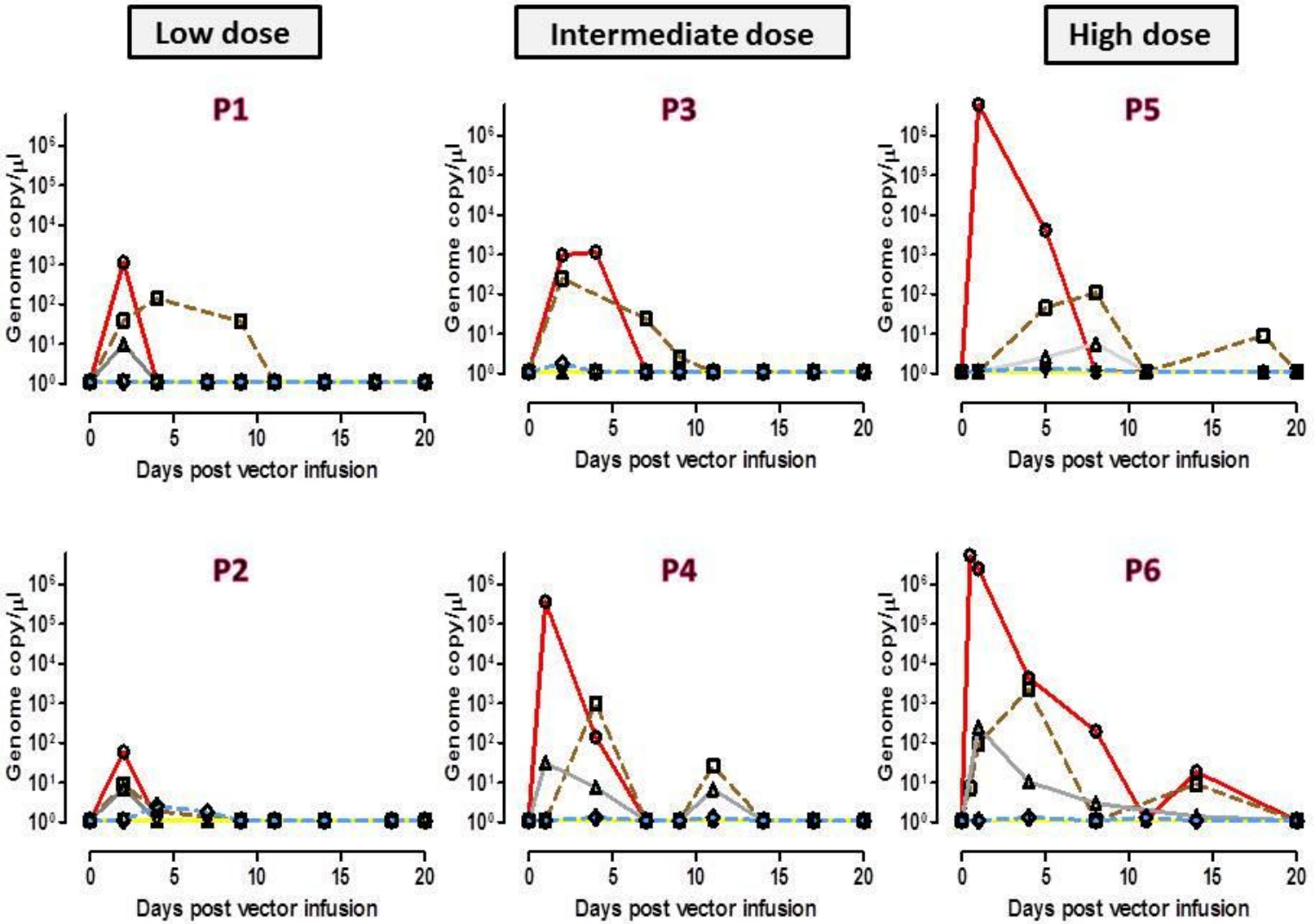
SUPPLEMENTARY TABLE 1.

SUMMARY OF ADVERSE EVENTS

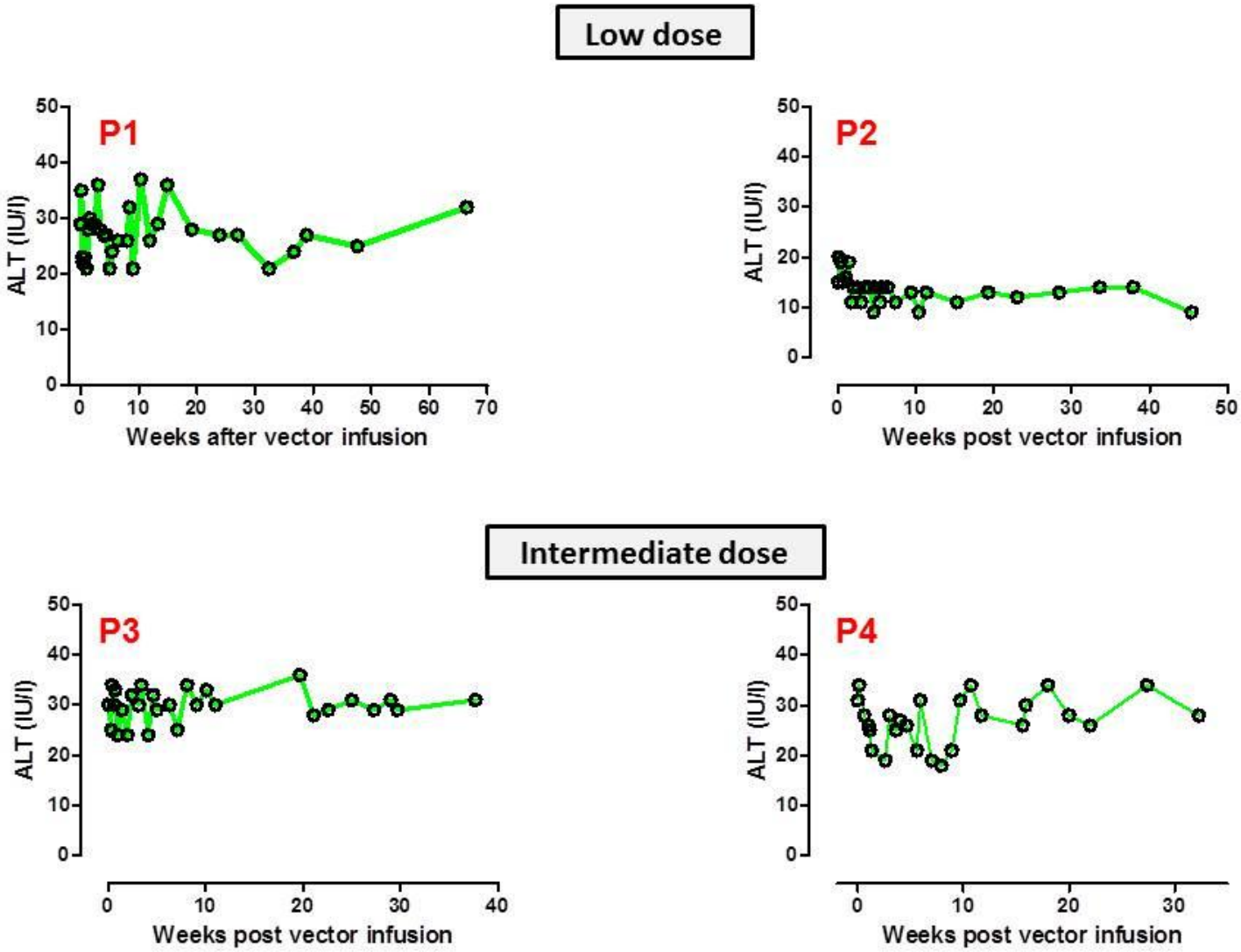
Participant	Description of event	Relationship to study agent
2	Subject 2 developed a normocytic normochromic anemia (trough Hb = 10.9g/dl) at 7 weeks after gene transfer, which was secondary to Staphylococcus capitis and Staphylococcus epidermidis infection of a loosened left artificial hip joint, implanted 20 years previously. He underwent a second left hip replacement under appropriate antibiotic and FIX concentrate cover with a gradual improvement of the anemia.	As the hip joint had begun to loosen prior to enrollment, this event was reported as a SAE unrelated to vector administration.
3	Participant 3 suffered a transient period of bradycardia, at 16 weeks after gene transfer when being prepared for left knee surgery to remove a metal plate left from an earlier osteotomy with tibial advancement, carried out for correction of deformity associated with hemophilic arthropathy. A Cardiology review concluded that the bradycardia was secondary to his use of a beta-blocker, which he had taken for several years for hypertension. The dose of the medication was adjusted	This event was deemed unrelated to vector administration

	and the participant has not suffered further arrhythmias.	
5	Participant 5 showed evidence of cyclic cytopenia during the trial. This was characterized by mild decrease in neutrophil (trough levels of $1.3x10^9$/l) and platelet (trough levels of $116x10^9$/l) count approximately every 21 days, lasting for an average of 5 days prior to returning to normal values.	This participant had a long history of cyclical cytopenia which had been document by his local hospital prior to his participation in the trial. This event was therefore considered to be unrelated to vector administration.
5	At week 7 after vector infusion, Participant 5 developed elevated levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which reached a peak of 143 and 202 IU/L at 58 days after vector infusion (upper limit of normal = 37 IU/l and 41 IU/l for AST and ALT respectively) normal range. This participant was commenced on a short course of corticosteroids and by week 10 post gene transfer, the liver enzymes had returned to the normal range.	This event was reported as a Grade III serious adverse event related to the study agent scAAV2/8-LP1-hFIXco.

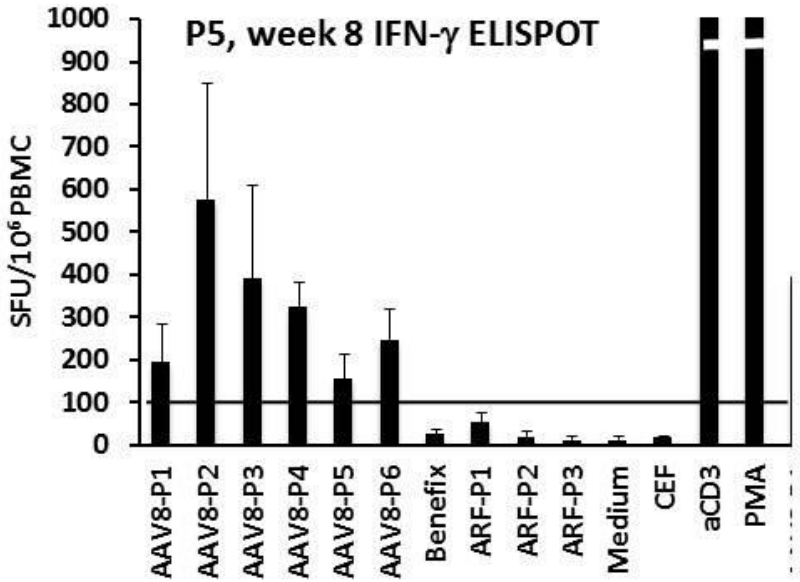
Supplemental Figure 1



Supplemental Figure 2



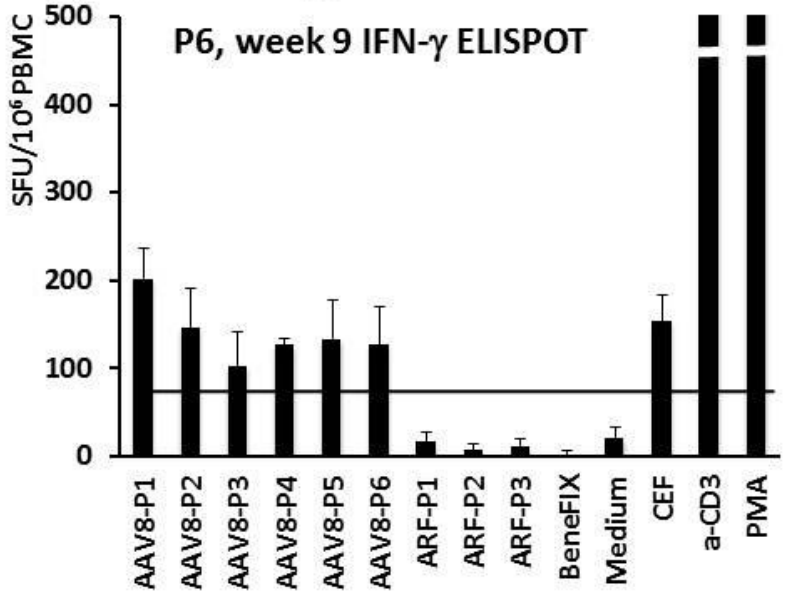
Supplemental Figure 3A



Pool 1		Pool 2		Pool 3		Pool 4		Pool 5		Pool 6	
1	2	3	4	5	6	7	8	9	10	11	12
13	14	15	16	17	18	19	20	21	22	23	24
25	26	27	28	29	30	31	32	33	34	35	36
37	38	39	40	41	42	43	44	45	46	47	48
49	50	51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	**70**	71	72
73	74	**75**	76	77	78	79	80	81	82	83	84
85	86	87	**88**	89	90	91	92	93	94	95	96
97	98	99	100	101	102	103	104	105	106	107	108
109	110	111	112	113	114	115	116	117	118	119	120
121	122	123	124	125	126	127	128	129	130	131	132
133	134	135	136	137	138	139	**140**	141	142	143	144
145	146										

HLA-A	HLA-B
02:01/04/07/09/17/18/20/24/25/29/30/31/33/42/49/59/60/64/66/67; 02:01/07/09/18/20/24/25/29/30/31/33/42/49/59/60/64/66/67.1,2	44:02/05/11/14/27/33/35; 44:02/11/27/33/35.1,2
HLA-A*0201 predicted binders: peptides 75 and 88	HLA-B*4402 predicted binders: peptides 70 and 140

Supplemental Figure 3B



Pool 1		Pool 2		Pool 3		Pool 4		Pool 5		Pool 6	
1	2	3	4	5	6	7	8	9	10	11	12
13	14	15	16	17	18	19	20	21	22	23	24
25	26	27	28	29	30	31	32	33	34	35	36
37	38	39	40	41	42	43	44	45	46	47	48
49	50	51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70	71	72
73	74	**75**	76	77	78	79	80	81	82	83	84
85	86	87	**88**	89	90	91	92	93	94	95	96
97	98	99	100	101	102	103	104	105	106	107	108
109	110	111	112	113	114	115	116	117	118	119	120
121	122	123	124	125	126	127	128	129	130	131	132
133	134	135	136	137	138	139	140	141	142	143	144
145	146										

HLA-A	HLA-B
02:01/01L/07/0918/20/24/25/29/30/31/33/34/42/59/62/66/67; 68:01/22/25.1,2	57:03; 50:01.1,2
HLA-A*0201 predicted binders: peptides 75 and 88	Not determined.